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원 저

The Effects of *Platycodi Radix* on the Induction of LPS and the Activation of NF-κBp, the Lung Disease of White Rats

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Objective & Methods : We examined the effects of *Platycodi radix* on the process of lipopolysaccharide (LPS)-induced nuclear factor (NF)-κBp65 and inhibitory (I)-κBα alteration in RAW 264.7 cells and acute lung injury in rats.

Results : Immunoblot analysis showed that LPS-induced degradation of I-κBα in RAW 264.7 was inhibited by pretreatment of *Platycodi radix*. The total cells of bronchoalveolar lavage fluid by LPS challenge markedly decreased in the *Platycodi radix* pretreatment rats. *Platycodi radix* pretreatment also caused a decline in neutrophils infiltration into interstitium of the lung. In the alveolar macrophages and neutrophils, decreased NF-κBp65 and inducible nitric oxide synthase and increased I-κBα immunoreaction were detected in *Platycodi radix* pretreated rats compared with LPS alone treated ones.

Conclusion : It may be concluded that *Platycodi radix* attenuates the development of LPS-induced inflammation by reduction of NF-κBp65 activation and neutrophil-mediated acute lung injury. *Platycodi radix* would be useful as a therapeutic agent for endotoxin-induced lung disease.

Key Words: *Platycodi Radix*, lipopolysaccharide(LPS), NF-κBp65, acute lung injury, rat

Introduction

The administration of lipopolysaccharide (LPS) present in the wall of gram-negative bacteria plays a major role in the release of several pro-inflammatory cytokines and these cytokines induce numerous effects involving fever, septic shock and death (Nathan and Xie[1] 1994; Bellezzo et al.[2], 1996). LPS induces various cytokines from macrophage and T-cell, which induce expression of inducible nitric oxide synthase (iNOS) mRNA or tumor necrosis factor (TNF)-α through the activation of transcriptional nuclear factor (NF)-κB (Ando et al.[3], 1998).

The NF-κB lie dormant in the cytoplasm of unstimulated cells and its activity is negatively regulated by a family of inhibitor protein known as inhibitory (I)-κB (Fels and Cohn[4], 1986; Siebenlist et al.[5], 1994; Kramer et al.[6], 1999; Mustafa et al.[7], 1999). Under the activated conditions, NF-κB is dissociated from I-κB and is translocated into the nucleus where it induces transcriptional up-regulation of various proinflammatory mediators that contribute to the systemic inflammatory response, such as TNF-α and interleukin-8 (Baeuerle and Baichwal[8], 1997; Kramer et al.[6], 1999).

Platycodi Radix was recorded in classic Korean

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medicine, Bang-Yak-Hap-Pyeon(fang yao he bian), (Hwang[9], 1996) and has been clinically used for the so-called a disease of respiratory system such as a soar throat, phlegm and pneumonia. *Platycodi Radix* has a wide application for the treatment of a disease, especially inflammation, of respiratory tract and lung as a complementary therapeutic agent.

Because LPS stimulation elicits an increase of NF-κB activation with corresponding degradation of its inhibitor I-κB (Kramer et al.[6], 1999; Yamakawa et al.[10], 1999), the prevention of NF-κB activation may be useful in the therapy of LPS-induced disorders (Ruetten and Thiemermann[11], 1997).

Thus blocking of NF-κB activation may be an effective strategy in the treatment of LPS-induced lung injury. We postulated that *Platycodi Radix* would attenuates lung injury following intraperitoneal challenge with LPS throughout its effect as an inhibitor of NF-κB expression. In the present study, we investigated the effects of *Platycodi Radix*, as a complementary therapeutic agent to lung disease, on the alteration of inflammatory proteins such as NF-κBp65, I-κBα and iNOS in the process of LPS-induced lung injury.

Materials and methods

1. Preparation of aqueous extract

The *Platycodi Radix* was prepared as follows : after drying, the herb, 14g of *Platycodi Radix* (Platycodon grandiflorum) was extracted with 1L of distilled water at 100℃ for 1 hours. The extract was filtered through 0.45㎛ filter, freeze-dried and kept at 4℃. The dried extract was dissolved in phosphate buffered saline (PBS) before use.

2. Reagents

Anti-iNOS rabbit polyclonal antibody was obtained from CALBIOCHEM (San Diego, CA). Rabbit polyclonal antibodies raised against NF-κBp65, I-κBα and TNF-α and horse radish peroxidase-conjugated anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Avidin-biotin-peroxidase complex kit and substrate kit for peroxidase were purchased from Vector Lab (Burlingame, CA) and LPS (phenol extracted Salmonella enteritidis) and other all reagents from Sigma (St. Louis, MO).

3. Cell culture and LPS and *Platycodi Radix* treatment

The RAW 264.7 cells, mouse macrophage cell line, were purchased from the KCLB (Korean Cell Line Bank, Seoul, Korea) and maintained in DMEM (Gibco BRL, Grand Island, NY) supplemented with 1% penicillin-streptomycin (Gibco BRL) and 10% fetal bovine serum (Gibco BRL) at 37℃ in 5% CO_2 enriched air. RAW 264.7 cells (2 X 106/dish) were transferred to 100mm polystyrene culture dish (Falcon, San Jose, CA) and stabilized for 24 hours. Cells were pretreated with 3mg/ml *of Platycodi Radix* for 12 hours. After pretreatment, cells were exposed to 500ng/ml of LPS for 90 minutes.

4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Platycodi Radix and LPS treated cells were harvested with police larva (Falcon), and then washed in cold PBS. The cells were lysed in lysis buffer (250mM NaCl, 25mM Tris-Cl pH7.5, 5mM EDTA pH8.0, 1% NP-40, 1mM PMSF and 5mM DTT) at 4℃ for 30 minutes. Protein concentrations were quantified using BioRad protein assay kit (BioRad Lab, Hercules, CA), following the procedure described by the manufacturer. 50mg of proteins were separated by 10% SDS-PAGE. The resulting gels were transferred to nitrocellulose

membranes, and then the membranes were blocked with 10% skim milk in PBS-T (0.1% Tween 20 in PBS) for 1 hour at room temperature. After blocking, the membranes were incubated with anti-NF-κBp65 and I-κBα antibodies at 4℃ for overnight. After washing in PBS-T three times, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibody and the antibody-specific proteins were visualized by the ECL detection system according to the recommended procedure.

5. Rat treatment and LPS and *Platycodi Radix* administration

Male Sprague-Dawley rats, weighing about 120g on average, were obtained from Taconic & SamYuk Co. in Korea. Rats were housed under conditioned of 22℃ and 12 hours dark and light cycle, and were fed a commercial diet and allowed tap water ad libitum starting 2 weeks before and throughout the study. Rats of the LPS alone and LPS plus *Platycodi Radix* group were administrated intraperitoneally 3 times, 24, 8 and 3 hours before LPS challenge, with either PBS or *Platycodi Radix* at a concentration of 100mg/Kg. After pretreatment, rats were challenged intraperitoneally with 6mg/Kg of LPS and control one with same volume of PBS. Rats were sacrificed at interval 3 and 6 hours after LPS challenge.

6. Bronchoalveolar lavage fluid and cell counts

For bronchoalveolar lavage fluid, the rats were anestherized with ether and a thoracotomy was performed. The lungs were lavaged with 15ml of sterile PBS. Cell counts were performed in bronchoalveolar lavage fluid on a hematocytometer.

7. Histopathology

The lung were fixed in 4% paraformaldehyde in PBS for 18 hours and dehydrated in a graded ethanol series. After embedded in paraffin, serial 5㎛ thick sections were prepared. For histopathological examinations, hematoxylin-eosin stain and periodic acid Schiffs (PAS) reaction were used.

8. Immunohistochemistry

After deparaffinized in 58℃ xylene, the sections were exposed for 30 minutes to 0.3% methanolic hydrogen peroxide, followed by washing with PBS. Tissues were then treated with goat normal serum at room temperature for 30 minutes followed by treatment with anti-NF-κBp65, I-κBα and iNOS diluted for 1:500 in moisture chamber for 16 hours at 4℃. After washed by PBS, tissues were incubated with the secondary antisera, biotinylated anti-rabbit IgG for 30 minutes, followed by washing with PBS. These sections were further incubated in avidin-biotin-peroxidase complex kit for 60 minutes at room temperature. Diaminobenzidine substrate kit for peroxidase was applied. For the controls, treatment with primary and secondary antibodies was omitted.

Results

1. Effects of *Platycodi Radix* on the LPS-induced activation of NF-κBp65 in RAW 264.7.

To examine the effect of *Platycodi Radix* on the LPS-induced inflammatory response, we examined alteration of inflammation-related proteins including NF-κBp65 and I-κBα. As a result, protein level of NF-κBp65 was increased by LPS treatment, but Platycodi Radix-pretreated cells were no effect. Since activation of NF-κBp65 is closely linked to degradation of negative regulator, I-κBα, we analyzed the level of I-κBα. As shown a panel of Fig. 1, LPS-induced degradation of I-κBα was inhibited by pretreatment of *Platycodi Radix*.

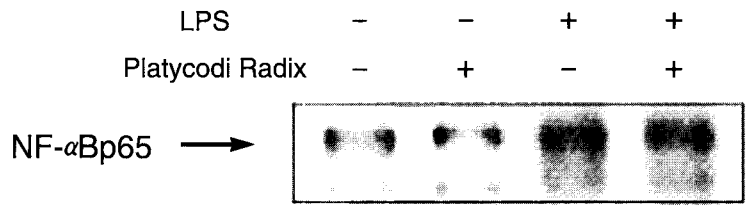

Fig. 1. Inhibitory effect of *Platycodi Radix* on the induction of NF-κBp65 and the degradation of I-κBα in RAW 264.7. Cells were pretreated with 3mg/ml of *Platycodi Radix* before LPS challenge (500ng/ml), and then exposed to LPS for 90 minutes. Sample was subjected to SDS-PAGE followed by the Western blot analysis using an NF-κBp65 and I-κBα antibody.

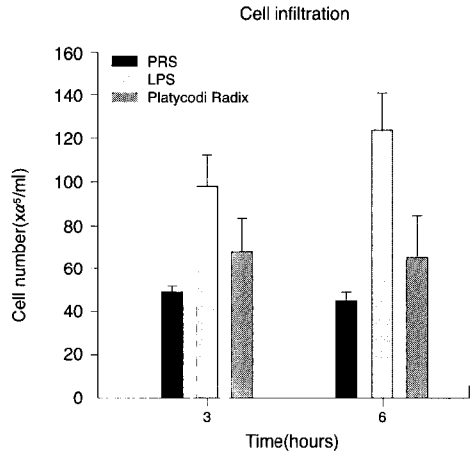

Fig. 2. Total cell of bronchoalveolar lavage fluid in rats treated with intraperitoneal LPS challenge (6mg/Kg). *p<0.001 for LPS alone treated rats compared with control ones and *p<0.01 for *Platycodi Radix*(100mg/Kg) pretreated rats for compared with LPS alone treated ones.

Platycodi Radix not only inhibits LPS-induced degradation of I-κBα, but also increases the basal level of I-κBα protein.

2. Cell counts in the bronchoalveolar lavage fluid

Fig. 2 shows the number of total cells in the bronchoalveolar lavage fluid 3 and 6 hours after treatment with LPS. The total cells of bronchoalveolar lavage fluid in the LPS alone treated rats markedly increased compared with control ones. However, *Platycodi Radix* pretreatment significantly attenuated, especially 6 hours after LPS challenge, the total cell numbers in the bronchoalveolar lavage fluid.

3. Histopathology

A mild inflammatory changes was observed in the rat lung from 3 hours after peritoneal LPS challenge. LPS shock caused a rise in neutrophils count and alveolar macrophage was also infiltrated into the interstitium and alveolar space. Although a few of neutrophils were infiltrated into the alveolar space, a severe neutrophilic

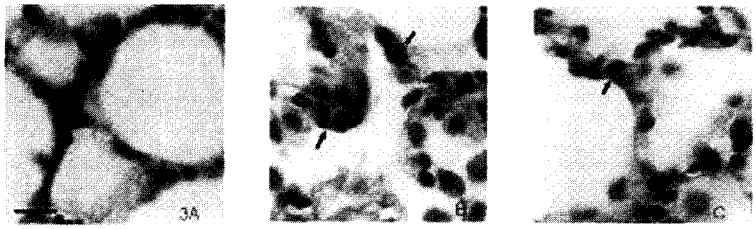

Fig. 3. The PAS reaction in the lung of normal (A), LPS (6mg/Kg) alone treated (B) and *Platycodi Radix*(100mg/Kg) pretreated rats (C) at 6 hours after LPS challenge. Note severe infiltration of neutrophils (arrows) into interstitium in the LPS challenged rats and a decline of neutrophils count in the *Platycodi Radix* pretreated rats. Scale bar = 30㎛.

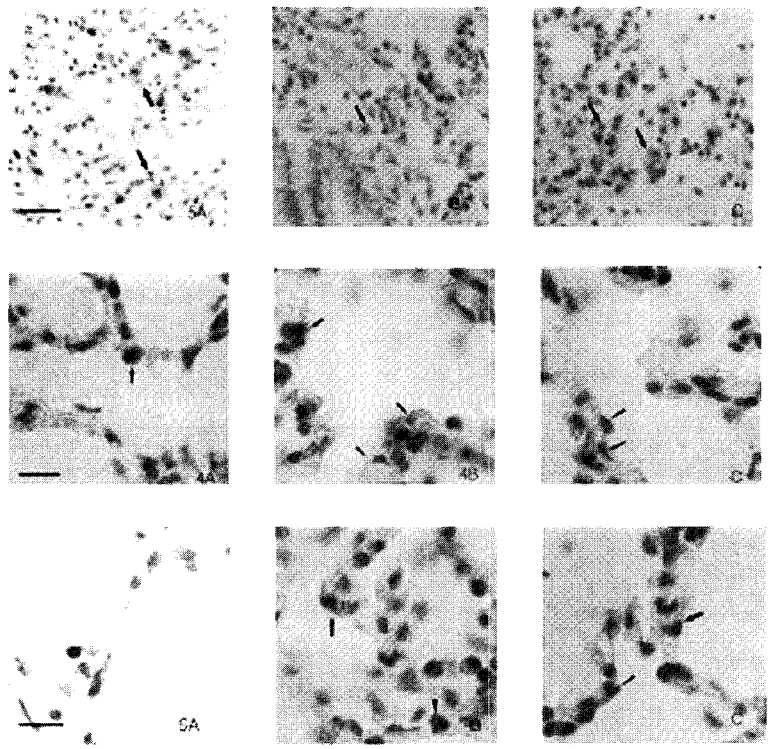

Fig. 4-6. Immunohistochemical localization of NF-κBp65 (Fig. 4), I-κBα (Fig. 5) and iNOS (Fig. 6) in the lung of normal (A), LPS (6mg/Kg) alone treated (B) and *Platycodi Radix*(100mg/Kg) pretreated rats (C) at 6 hours after LPS challenge. *Platycodi Radix* pretreated rats showed a decrease of NF-κBp65 and iNOS immunoreaction in the alveolar macrophage (arrows) and infiltrated neutrophils (arrow heads) and an increase of I-κBα immunoreaction in the macrophage. Scale bar = 30㎛.

alveolitis was not detected in LPS challenged rats. The slight decline of alveolar macrophages and neutrophils infiltration was observed in the *Platycodi Radix* pretreated rats (Fig. 3).

4. Immunohistochemistry

The results of the immunohistochemical study on the inflammation-related protein shows Figs. 4-6. NF-κ Bp65, I-κBα and iNOS expression were mainly detected

in the alveolar macrophages and neutrophils in the lung and more intensive expression was observed in the alveolar macrophages. The number of alveolar macrophages and neutrophils showing NF-κBp65, I-κB α and iNOS immunoreaction, especially neutrophils, was increased in the LPS treated rats. Enhanced immunoreaction for NF-κBp65 and iNOS were also observed in rats challenged with LPS. With a slight decline of immunoreactive cells in number, decreased NF-κBp65 and iNOS and increased I-κBα immunoreaction were demonstrated in these cells of *Platycodi Radix* pretreated rats.

Discussion

Alveolar macrophages, the key lung cells in host defence, are considered to play a central role in the regulation of the immune response to inhaled pathogens and development of inflammation (Fels and Cohn[4], 1986; Li et al.[12], 1999). Under stimulated conditions by LPS challenges, macrophages are a major source of inflammatory cytokines and its cytokine release is predominantly regulated by the transcriptional rates of cytokines gene.

NF-κB is a primary transcription factor for inflammatory cytokines and its activation is also known to produce inflammatory enzymes (Hong et al.[13], 1999; Rothwarf and Karin[14], 1999). NF-κB activation at the end of LPS shock indicates a primed alveolar macrophage (Blackwell and Christman[15], 1997; Fan et al.[16], 1998). Because LPS-induced I-κB degradation is closely related to NF-κB activation, inhibition of NF-κB activation with an inhibitor of I-αB degradation eliminate TNF-α synthesis and the expression of iNOS in the LPS-stimulated cells (Díaz-Guerra et al.[17], 1996; Iimuro et al.[18], 2000).

Therefore we postulated that agents that prevent activation of NF-κB in macrophages could prevent propagation of the inflammatory cascade. When we examined the effect of *Platycodi Radix* on alteration of inflammation-related proteins involving NF-κBp65 and I-κBα in LPS treated RAW 264.7 cell, LPS-induced degradation of I-κBα was inhibited by pretreatment of *Platycodi Radix* and the basal level of I-κBα protein also increased. It suggests that LPS-induced inflammation was regulated by *Platycodi Radix* through increase of the I-κBα level and inhibition of its degradation pathway in mouse macrophage cell line.

With inflammatory cytokines, neutrophils are a principal cellular mediator in the development of acute lung injury. The influx of activated neutrophils into the lungs are thought to be important in the pathogenesis of lung injury (Nathens et al.[19], 1997). Although several chemotactic factors for neutrophils have been identified, NF-κB is thought to be important in the gene expression of all neutrophilic chemotactic cytokines (Blackwell et al.[20], 1997). Intense neutrophils influx is closely associated with NF-κB activity in the lung tissue (Blackwell and Christman[15], 1997; Blackwell et al.[21], 1999).

So various anti-neutrophilic chemotactic chemokines strategies appear to attenuate acute lung injury. Intraperitoneal LPS injection does not produce significant neutrophilic alveolitis in the first few hours after LPS challenge, but results in systemic inflammation involving inflammatory cells infiltrated in the interstitium and air space of the lung (Blackwell et al.[22], 1994; Carter et al.[23], 1998).

The activation of NF-κB is also associated with the production of inflammatory enzymes iNOS. Enhanced formation of NO by iNOS plays also a critical role in the pathogenesis of LPS-caused acute inflammation as well as dysfunction (Ruetten and Thiemermann[11], 1997; Numata et al[24]. 1998).

In the present total cells studies, we observed that *Platycodi Radix* pretreatment showed a statistically

significant decreases in the total cell of bronchoalveolar lavage fluid. Especially, as for the histophatological studies, *Platycodi Radix* pretreatment rats showed a decline count of infiltrated neutrophils into interstitium compared with LPS alone challenged rats. And in the immunohistochemical studies, different immunoreactivity between *Platycodi Radix* pretreated rats and LPS alone treated ones were demonstrated in the alveolar macrophages and neutrophils.

Decreased immunoreaction for NF-κBp65 and iNOS and increased one for I-κBα were observed in the *Platycodi Radix* pretreatment rats.

Both negative regulation of NF-κB activity in response to specific signals and neutrophils infiltration into the interstitium are very complex process. However, *Platycodi Radix* pretreatment inhibited the degradation of I-κBα in mouse macrophage cell line and showed a decrease of NF-κ Bp65 and iNOS immunoreaction in the alveolar macrophages and neutrophils in the lung tissue.

Platycodi Radix may act as an inhibitor of NF-κB activation through the inhibition of I-κB degradation and then have an influences on a decline of iNOS production. Decreased intensity of neutrophilic infiltration in the *Platycodi Radix* pretreated rats are may due to decreased NF-κB activity in the lung. Therefore it may be concluded that *Platycodi Radix* would be useful as a therapeutic agent for inflammatory disease of respiratory system.

Conclusion

We examined the effects of *Platycodi Radix* on the process of lipopolysaccharide (LPS)-induced nuclear factor (NF)-κBp65 and inhibitory (I)-κBα alteration in RAW 264.7 cell and acute lung injury in rats.

The results were as follows;

1. *Platycodi Radix* decreased the NF-κB level and increased the I-κBα level in RAW 264.7 cell with LPS-induced inflammation.
2. *Platycodi Radix* pretreatment decreased in the total cell of bronchoalveolar lavage fluid.
3. In the histophatological studies, *Platycodi Radix* pretreatment rats showed a decline count of infiltrated neutrophils into interstitium compared with LPS alone challenged rats.
4. In the immunohistochemical studies, decreased immunoreaction for NF-κBp65 and iNOS and increased one for I-κBα were observed in the *Platycodi Radix* pretreatment rats.

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